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Original article

Serum and milk concentrations of oxidant and anti-oxidant markers in dairy cows affected with bloody milk

Z. Bani Ismail, S.M. Abutarbush, K. Al-Qudah, F. Omoush

Department of Veterinary Clinical Sciences, Faculty of Veterinary Medicine, Jordan University of Science and Technology, Irbid 22110, Jordan

Abstract

This study was conducted to determine the serum and milk levels of thiobarbturic acid-reactive substances (TBARS), nitric oxide (NO), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), vitamin E and selenium, IL-4 and IL-6 in lactating dairy cows affected with bloody milk using commercially available ELISA kits. Milk and whole blood samples were collected from 60 cows affected with bloody milk and 20 apparently healthy cows for control. In the serum, levels of GSH-Px and SOD were significantly ($p<0.05$) higher in healthy cows compared to cows affected with bloody milk while the levels of TBARS and NO were significantly ($p<0.05$) higher in affected cows. In the milk, levels of SOD, TBARS and NO were significantly ($p<0.05$) higher in affected cows. In the serum, levels of vitamin E were significantly ($p<0.05$) lower in affected cows compared to healthy cows, while no significant changes were observed in the levels of this vitamin in the milk between healthy and affected cows. In the serum, levels of selenium were significantly ($p<0.05$) lower in affected cows while in milk, selenium levels were significantly ($p<0.05$) higher in affected cows compared to healthy ones. Levels of IL-4 were significantly ($p<0.05$) lower in the serum and milk of affected cows compared to healthy cows while levels of IL-6 were significantly ($p<0.05$) higher in both serum and milk of affected cows. Results of this study suggest a possible role of oxidative stress in the pathogenesis of bloody milk in dairy cows.

Key words: dairy cows, oxidative stress, anti-oxidants, dietary supplements, bloody milk

Correspondence to: Z. Bani Ismail, e-mail: zuhair72@just.edu.jo

Introduction

Bloody milk (hemolactia) is a fairly common condition affecting lactating dairy cows worldwide (Bani Ismail 2016). Bloody milk can be associated with significant economic losses due to decreased production, discarded milk, cost of veterinary consultation and treatment (Bani Ismail 2016). The condition occurs sporadically, but in some geographic locations, the incidence rate was estimated to reach up to 50% (Bani Ismail 2016). While in heifers the condition is frequently considered physiological in origin, the etiology remains uncertain in multiparous cows (Bani Ismail 2016).

Oxidative stress was found to play an important pathological role in several diseases of dairy cows including ketosis, abomasal displacement, clinical and subclinical mastitis, anestrous and infertility, metritis, retained placenta, enteritis, pneumonia, omphalitis and impaired immune responses in the periparturient period (Al-Qudah 2009, Jovanović et al. 2013, Cigliano et al. 2014, Konvicna et al. 2015, Ibrahim et al. 2016, Sharma et al. 2016, Du et al. 2017, Anil and Meenaxi 2018, Batistel et al. 2018, Mahapatra et al. 2018, Fiore et al. 2019, Mayasari et al. 2019, Yurdakul and Aydogdu 2019, Zigo et al. 2019).

During the immediate post parturient period, the mammary gland tissues are placed under tremendous physiological stress and increased energy and oxygen demand (Cigliano et al. 2014, Konvicna et al. 2015). This could lead to excessive release of reactive oxygen substances (ROS) inducing significant tissue damage. Currently, there are no published scientific data regarding the relationship between the incidence of bloody milk and oxidative stress/anti-oxidents levels in dairy cows. Therefore, the objective of this study was to assess the levels of oxidative stress and anti-oxidative defense mechanisms in cows with bloody milk by the assessment of thiobarbturic acid-reactive substances (TBARS), nitric oxide (NO), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), vitamin E and selenium, IL-4 and IL-6 in lactating dairy cows affected with bloody milk.

Materials and Methods

Ethical approval

All procedures and protocols performed in this study were approved by the animal care and use committee of Jordan University of Science and Technology (ACUC-JUST). Written consent was signed by the owner of the dairy farm from which animals were used in the study.

Herd management

The study was conducted as part of a herd investigation of increased incidence of bloody milk involving 1 dairy farm. The farm consisted of 2100 lactating Holstein dairy cows located in Al-Dulail region in the Northern East of Jordan. The study was conducted during the summer of 2019 between the months of April and September when the incidence of the condition was reportedly highest. The cows in the farm were housed freely in open sheds. The cows were fed total mixed ration (TMR) 3 times per day with ad lib of fresh water. The cows were milked 3 times per day in a double 56-cow herringbone-milking parlor.

Animals and selection criteria

Samples were collected from 60 cows diagnosed with bloody milk. For control, samples were collected also from 20 apparently healthy cows in a similar age and stage of lactation. In the affected cow group, cows were selected for the study based on the clinical diagnosis of bloody milk at any stage of lactation. The farm records of selected cows in the affected and control groups were reviewed and cow data regarding parity, days-in-milk (DIM), and previous medical history was collected.

Affected cows were subjected to a complete physical examination by the principal investigator. Physical examination included rectal temperature, heart rate, respiration rate, mucous membrane color, and rumen motility. Examination of the udder included palpation to detect signs of inflammation such as redness, heat, pain and swelling. Milk secretion was examined for consistency, color and presence of blood or milk clots.

To be enrolled in the study, cows in the affected group must have a diagnosis of bloody milk in one or more quarters with negative CMT and negative milk bacterial culture and no antibiotics administered locally or systemically within the last 7 days prior to the diagnosis.

In the control group, cows must be apparently healthy, CMT negative with negative milk bacterial culture and no history of antibiotic administration within the last 7 days prior to the initiation of the study.

Environmental assessment

A thorough environmental examination was performed to determine possible sources of trauma. Other investigations such as multiple bacterial cultures of milk samples were performed to rule out mastitis, hematology and biochemistry including coagulation profiles were performed to rule out systemic infections and coagulation abnormalities (Bani Ismail 2016).

Table 1. Serum and milk concentrations (Mean±SD) of glutathione peroxidase, superoxide dismutase, TBARS, and nitric oxide in apparently healthy cows and cows affected with bloody milk.

Parameters	Healthy cows (N=20)	Affected cows (N=60)
Serum		
Glutathione peroxidase (pg/ml)	4953±880	3567±950*
Super oxide dismutase (pg/ml)	4830±640	3131±710*
TBARS (ng/ml)	884±185	1203±350*
Nitric oxide (µmol/l)	27±2.5	54±6*
Milk		
Glutathione peroxidase (pg/ml)	1954±718	2329±644
Superoxide dismutase (pg/ml)	1700±173	3113±135*
TBARS (ng/ml)	336±88	600±135*
Nitric oxide (µmol/l)	3.67±0.75	7.5±0.95*

* $p<0.05$

Sample collection

Whole blood samples were collected via coccygeal vein puncture and placed in plain blood tubes. Blood samples were allowed to clot in room temperature, centrifuges at 5000 g for 10 minutes and serum was transferred into Eppendorf tubes, identified and stored at -20°C until analysis was performed.

Approximately, 5 ml of milk samples were collected aseptically from each affected quarter and placed in sterile tubes for bacterial culture (Metzger et al. 2018). Another 50 ml, non-sterile milk samples were collected from each affected quarter for oxidant/antioxidants biomarkers determination. Similar milk samples were also collected from cows in the control group. Milk samples were transported to laboratory in an ice box within 4 hours. Milk samples were stored at -20°C until analysis was performed. In the laboratory, skimmed milk samples were used to determine various parameters. Skimmed milk was obtained by centrifugation of thawed milk samples at 5000 g for 10 minutes. The supernatant was obtained and stored at -20°C until analysis was performed.

Laboratory analysis

The serum and milk α-tocopherol and selenium (as selenium-binding protein 1) concentrations were assessed using commercially available ELISA kits (Mybiosource, USA) according to manufacturer's instructions (Jovanović et al. 2013). The microplates were read at 533 nm and 450 nm wavelengths, respectively using spectrophotometer (Thermo Fisher, USA). The intra and inter CV of the kit were <10% and the recovery rate was 95-98%.

The serum and milk concentrations of TBARS (Cayman Chemical, USA), NO, SOD, GSH-Px, IL-4 and IL-6 (Mybiosource, USA) were determined using commercially available ELISA kits according to manufacturer's instructions (Ibrahim et al. 2016). The microplates were read at 412-550 nm wavelength using spectrophotometer (Thermo Fisher, USA). The intra and inter CV of the kits were <10%. The recovery rate was 92%-98%.

Bacterial culture

Milk samples from healthy and affected cows were cultured using routine laboratory methods (Bani Ismail 2016). Briefly, milk samples were vigorously shaken and 50 µL from each milk sample was spread onto 5% sheep blood agar and MacConkey agar (Oxoid, UK) plates.

Statistical analysis

Data were reported as mean ± SD. One-way ANOVA followed by Tukey's post-test was used to compare the mean values of all measured parameters between healthy cows and cows affected with bloody milk. The correlation coefficient was determined to detect possible relationships between different parameters using linear regression analysis. Differences were considered statistically significant at $p<0.05$. Statistical analysis was performed using SPSS statistical software version 23 (IBM SPSS Statistics Software, Chicago, USA).

Results

In affected cows, the mean ± SD of parity and DIM were 2.9±1.8 and 177±145, respectively while in the control group, the mean ± SD of parity and DIM were 3.5±2.0 and 203±133, respectively. Physical examination of affected cows and control cows revealed no abnormalities involving any body system including normal rectal temperature, respiration rate, heart rate and rumen motility patterns. In affected cows, there was

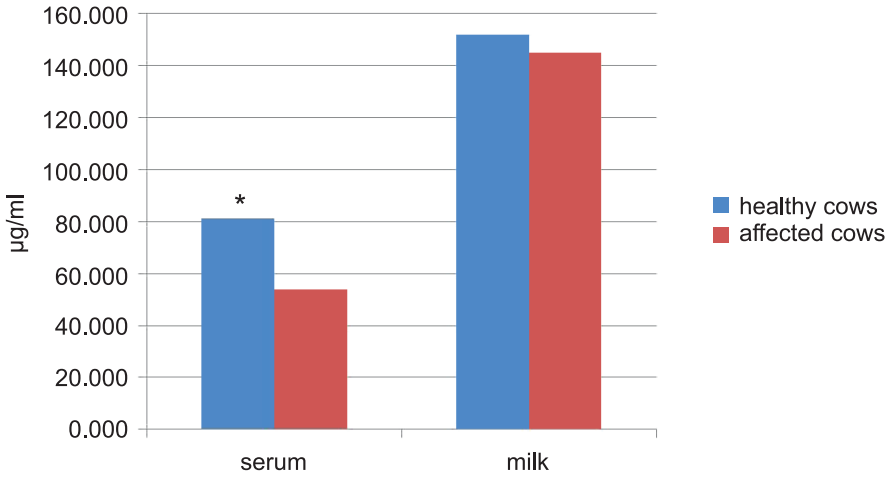

Fig. 1. Serum and milk concentrations of vitamin E (µg/ml) in healthy cows (N=20) and cows affected with bloody milk (N=60).
* p<0.05

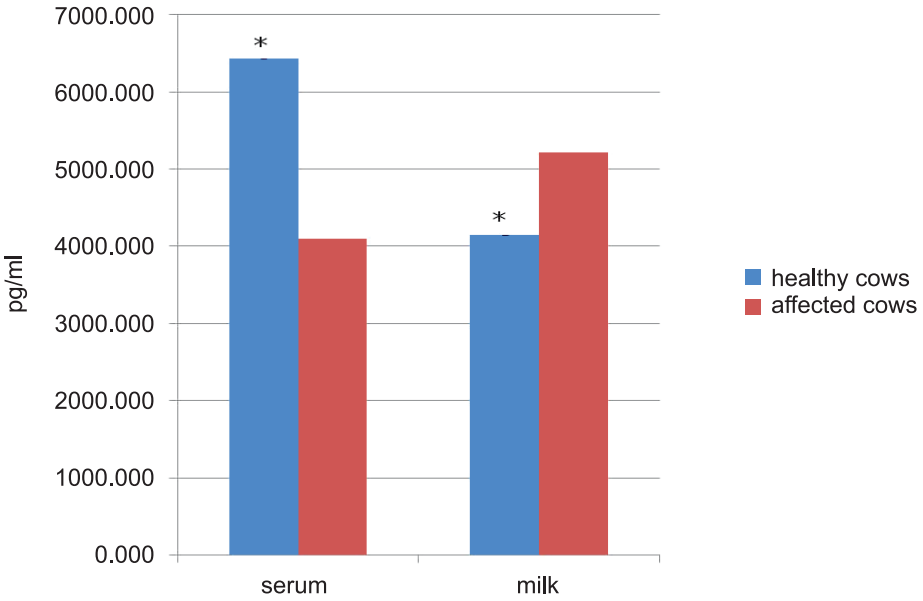

Fig. 2. Serum and milk concentrations of selenium (pg/ml) in healthy cows (N=20) and cows affected with bloody milk (N=60).
* p<0.05

no swelling, heat or pain detected during udder and teat examination. The milk secretion was blood-tinged involving one or more quarters in all affected cows (1 quarter in 33 cows, 2 quarters in 15 cows, 3 quarters in 9 cows, and 4 quarters in 3 cows). In control cows both the udder, teats and secretions were apparently normal. All cows in the affected and control group were CMT negative and bacterial culture revealed no growth.

The serum and milk (mean ± SD) concentrations of various oxidants and anti-oxidant parameters in healthy cows and cows affected with bloody milk are presented in Table 1. In the serum, levels of anti-oxidants enzymes GSH-Px and SOD were significantly ($p<0.05$) higher in healthy cows compared to cows affected with bloody milk while the levels of oxidative stress markers TBARS and NO were significantly ($p<0.05$) higher in the serum of affected cows. In milk, levels of GSH-Px and SOD were higher in affected cows, but significant difference ($p<0.05$) was only observed in values of SOD. The concentrations of TBARS and NO were also significantly ($p<0.05$) higher in the milk of affected cows.

Levels of vitamin E in the serum and milk of healthy cows and cows affected with bloody milk are presented in Fig. 1. Serum levels of vitamin E were significantly ($p<0.05$) lower in affected cows (54±11 µg/ml) compared to healthy cows (81.2±16 µg/ml), while no significant changes were observed in the levels of this vitamin in the milk between healthy (152±16 µg/ml) and affected cows (154±19 µg/ml).

Levels of selenium in serum and milk in healthy cows and cows affected with bloody milk are presented in Figure 2. Serum levels of selenium were significantly ($p<0.05$) lower affected cows with bloody

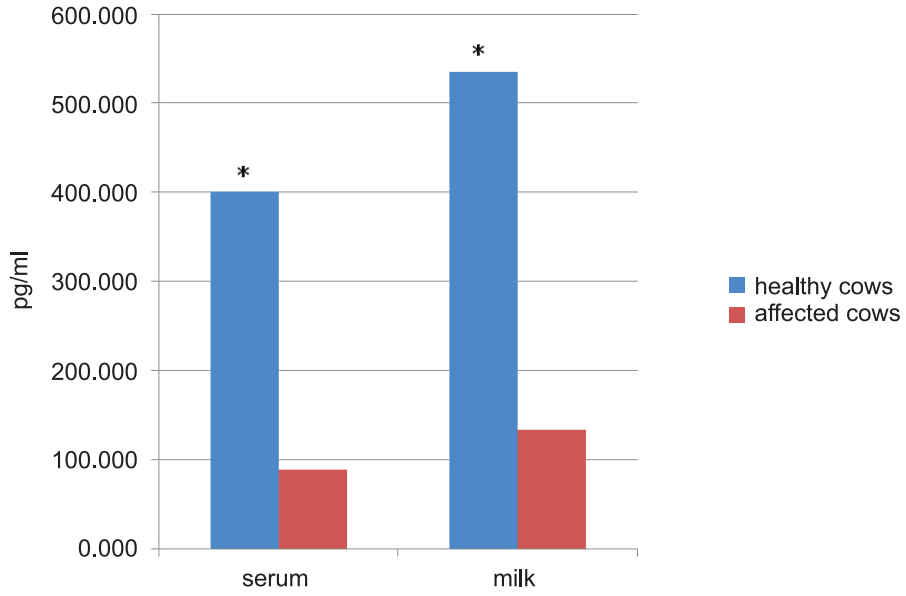

Fig. 3. Serum and milk concentrations of IL-4 (pg/ml) in healthy cows (N=20) and cows affected with bloody milk (N=60).
* p<0.05

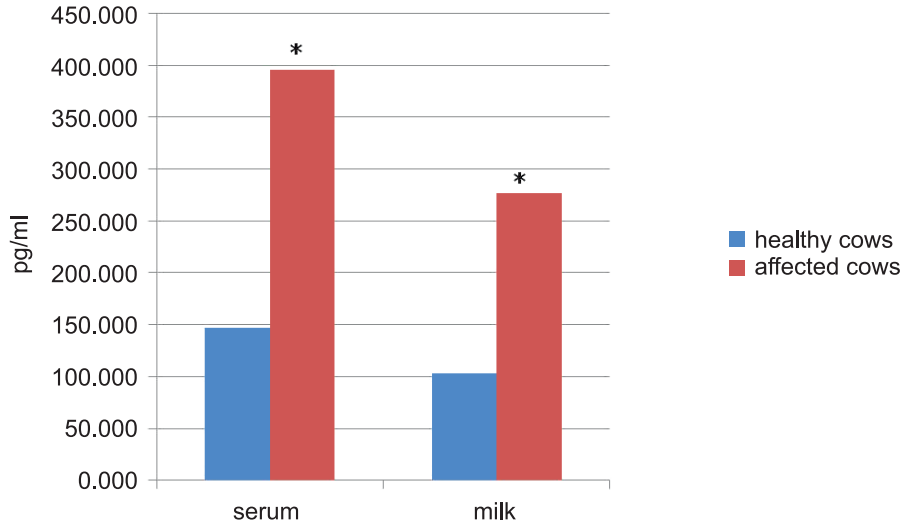

Fig. 4. Serum and milk concentrations of IL-6 (pg/ml) in healthy cows (N=20) and cows affected with bloody milk (N=60).
* p<0.05

milk (4100±1200 pg/ml) compared to healthy cows (6432±1600 pg/ml) while in milk, selenium levels were significantly (p<0.05) higher in affected cows (5210±1250 pg/ml) compared to healthy ones (4144±985 pg/ml).

Levels of IL-4 were significantly (p<0.05) lower in the serum and milk of affected cows (89±34 pg/ml and 134±85 pg/ml, respectively) compared to healthy cows (400±123 pg/ml and 535±112 pg/ml) (Fig. 3).

Levels of IL-6 however were significantly (p<0.05) higher in both serum and milk of affected cows (396±65 pg/ml and 277±89 pg/ml, respectively) compared to those values in healthy cows (147±33 pg/ml and 103±15 pg/ml, respectively) (Fig. 4).

Analysis of correlation coefficient revealed a significant (p<0.05) positive correlation between milk concentrations of TBARS (r=0.980), NO (r=0.880), and SOD (r=0.765) while a significant (p<0.05) negative correlation (r=-0.870) was observed between milk and serum values of GSH-Px and SOD. There was a significant (p<0.05) positive correlation between milk concentrations of vitamin E (r=0.790), and selenium (r=0.750). There was a significant (p<0.05) positive correlation between serum (r=0.856) and milk (r=0.666) concentrations of IL-6 while a significant (p<0.05) negative correlation was detected between milk (r=-0.740) and serum (r=-0.850) concentrations of IL-4.

Discussion

The exact cause and pathogenesis of bloody milk in dairy cows has not been determined yet. In one study, affected cows showed no abnormalities in the hematology, serum biochemistry, or the coagulation profiles (Bani Ismail 2016). In congruent with previous studies, bloody milk in the current study does not appear to be associated with clinical mastitis (Bani Ismail 2016). This was verified by negative bacterial culture of all milk samples from affected cows enrolled in the study. In addition, physical examination did not reveal any degree of udder edema in any of the affected cows. Udder edema is commonly diagnosed in high producing cows around parturition (Bani Ismail 2016). The average DIM in cows enrolled in this study was 177 and therefore, physiologic udder edema and infectious mastitis were ruled out as a likely cause of bloody milk in this study.

Previous studies in healthy multiparous Holstein cows indicated that levels of serum selenium, MDA, and GSH-Px varied according to the stage of lactation (Konvičná et al. 2015, Gong and Xiao 2016). It was reported that serum levels of selenium, MDA, and GSH-Px were significantly higher in cows in early lactation compared to cows at peak lactation and those in the dry period. It is well known that lactating cows, especially during the first 100 DIM are subjected to major physiological challenges due to negative energy balance, immune depression, and oxidative stress. Indeed, several clinical trials indicated significant decrease in the incidence of post parturient diseases after supplementing cows with selenium and vitamin E (Jovanović et al. 2013, Durgut et al. 2016, Mehdi and Dufrasne 2016, Omur et al. 2016, Khatti et al. 2017, Schäfers et al. 2018, Khalili et al. 2019).

It was found that cows in early and peak lactation have decreased total anti-oxidant capacity along with increased oxidative damage (Gong and Xiao 2016, Batistel et al. 2018). Similarly, in this study, the serum levels of oxidative stress markers TBARS and NO were significantly ($p<0.05$) higher in the serum and milk of affected cows compared to healthy cows while levels of the anti-oxidant enzymes GSH-Px and SOD were significantly ($p<0.05$) higher in healthy cows. In addition, serum levels of vitamin E were significantly ($p<0.05$) lower in affected cows compared to healthy cows. Moreover, serum levels of selenium were significantly ($p<0.05$) lower in cows affected with bloody milk while in milk, selenium levels were significantly ($p<0.05$) higher. These results further indicate the importance of vitamin E and selenium supplementation to dairy cows during different stages of lactation.

Various pro-inflammatory cytokines including IL-4 and IL-6 are produced in the body in response to infectious and non-infectious inflammatory stimuli (Kushibiki 2011). The release of pro-inflammatory cytokines in high concentrations is known to be associated with anorexia around parturition and therefore have been used as potential indicators of several postpartum conditions in dairy cows (Trevisi et al. 2010). In one study, significantly higher levels of IL-4 were reported in the serum and milk of cows affected with metabolic and reproductive conditions including mastitis, anorexia and reduced milk production in early lactation (Trevisi et al. 2015). Similarly, in this study, levels of IL-6 were significantly ($p<0.05$) higher in both serum and milk in cows affected with bloody milk compared to those values in healthy cows while levels of IL-4 were significantly ($p<0.05$) lower in the serum and milk of affected cows compared to healthy ones. Similar results were also reported in cows with clinical and subclinical mastitis (Ibrahim et al. 2016, Bochniarz et al. 2017).

In this study, there is a significant positive correlation between milk concentrations of TBARS, SOD and bloody milk, while a significant negative correlation was observed between bloody milk and serum values of GSH-Px and SOD. In addition, a significant positive correlation between milk concentrations of vitamin E and selenium and bloody milk was observed. These results are similar to previously reported correlations between clinical mastitis which indicated that the antioxidant defense mechanisms in dairy cows with acute mastitis are impaired leading to a significant oxidative stress and damage. It was concluded that the alterations in the antioxidant trace elements and pro-inflammatory cytokines are considered very reliable indicators of increased oxidative stress and tissue damage in dairy cows with acute clinical mastitis (Ibrahim et al. 2016).

The relationship between the occurrence of bloody milk and oxidative stress/anti-oxidant levels in serum and milk has not been studied before. The theory presented in this study was that oxidative stress plays an important role in the pathogenesis of bloody milk in dairy cows. The results of the study shed light on the importance of vitamin E and selenium supplementation to dairy cows during peak lactation to prevent this economically important condition.

References

Al-Qudah KM (**2009**) Oxidative stress in calves with acute or chronic bronchopneumonia. Revue Méd Vét 160: 231-236.

Anil M, Meenaxi S (**2018**) A review: oxidative stress during lactation in dairy cattle. Dairy and Vet Sci J 5: 555669.

Bani Ismail Z (**2016**) Successful treatment of bloody milk not associated with physiologic udder edema in lactating Holstein dairy cows using intravenous formalin administration. Bull UASVM Vet Med 73: 153-161.

Batistel F, Arroyo JM, Garces CI, Trevisi E, Parys C, Ballou MA, Cardoso FC, Loor JJ (**2018**) Ethyl-cellulose rumen-protected methionine alleviates inflammation and oxidative stress and improves neutrophil function during the periparturient period and early lactation in Holstein dairy cows. J Dairy Sci 101: 480-490.

Bochniarz M, Zdzisińska B, Wawron W, Szczubiał M, Dąbrowski R (**2017**) Milk and serum IL-4, IL-6, IL-10, and amyloid A concentrations in cows with subclinical mastitis caused by coagulase-negative staphylococci. J Dairy Sci 100: 9674–9680.

Cigliano L, Strazzullo M, Rossetti C, Grazioli G, Auriemma G, Sarubbi F, Iannuzzi C, Iannuzzi L, Spagnuolo MS (**2014**) Characterization of blood redox status of early and mid-late lactating dairy cows. Czech J Anim Sci 59: 170-181.

Du X, Shi Z, Peng Z, Zhao C, Zhang Y, Wang Z, Li X, Liu G, Li X (**2017**) Acetoacetate induces hepatocytes apoptosis by the ROS-mediated MAPKs pathway in ketotic cows. J Cell Physiol 232: 3296-3308.

Durgut R, Sagkan Ozturk A, Ozturk OH, Guzel M (**2016**) Evaluation of oxidative stress, antioxidant status and lipid profile in cattle with displacement of the abomasum. Ankara Üniv Vet Fac Derg 63: 137-141.

Fiore F, Spissu N, Sechi S, Cocco R (**2019**) Evaluation of oxidative stress in dairy cows with left displacement of abomasum. Animals (Basel) 9: 966.

Gong J, Xiao M (**2016**) Selenium and antioxidant status in dairy cows at different stages of lactation. Biol Trace Elem Res 171: 89-93.

Ibrahim HM, El-Seedy YY, Gomaa NA (**2016**) Cytokine response and oxidative stress status in dairy cows with acute clinical mastitis. J Dairy Vet Anim Res 3: 00064.

Jovanović IB, Veličković M, Vuković D, Milanović S, Valčić O, Gvozdić D (**2013**) Effects of different amounts of supplemental selenium and vitamin E on the incidence of retained placenta, selenium, malondialdehyde, and thyronines status in cows treated with prostaglandin F2α for the induction of parturition. J Vet Med 2013: 867453.

Khalili M, Chamani M, Amanlou H, Nikkhah A, Sadeghi A (**2019**) The effect of feeding inorganic and organic selenium sources on the performance and content of selenium in milk of transition dairy cows. Acta Sci Anim Sci 41: e44691.

Khatti A, Mehrotra S, Patel PK, Singh G, Maurya VP, Mahla AS, Chaudhari RK, Das GK, Singh M, Sarkar M, Kumar H, Krishnaswamy N (**2017**) Supplementation of vitamin E, selenium and increased energy allowance mitigates the transition stress and improves postpartum reproductive performance in the crossbred cow. Theriogenology 104: 142-148.

Konvicna J, Vargova M, Paulíkova I, Kovac G, Kostecka Z (**2015**) Oxidative stress and antioxidant status in dairy cows during prepartal and postpartal periods. Acta Vet Brno 84: 133-140.

Kushibiki S (**2011**) Tumor necrosis factor-α-induced inflammatory responses in cattle. Anim Sci J 82: 504-511.

Mahapatra A, Panigrahi S, Patra RC, Rout M, Ganguly S (**2018**) A Study on bovine mastitis related oxidative stress along with therapeutic regimen. Int J Curr Microbiol App Sci 7: 247-256.

Mayasari N, Trevisi E, Ferrari A, Kemp B, Parmentier HK, Van Knegsel AT (**2019**) Relationship between inflammatory biomarkers and oxidative stress with uterine health in dairy cows with different dry period lengths. Transl Anim Sci 3: 607-619.

Mehdi Y, Dufrasne I (**2016**) Selenium in cattle: a review. Molecules 21: 545.

Metzger SA, Hernandez LL, Skarlupka JH, Suen G, Walker TM, Ruegg PL (**2018**) Influence of sampling technique and bedding type on the milk microbiota: results of a pilot study. J Dairy Sci 101: 6346-6356.

Omur A, Kirbas A, Aksu E, Kandemir F, Dorman E, Kaynar O, Ucar O (**2016**) Effects of antioxidant vitamins (A, D, E) and trace elements (Cu, Mn, Se, Zn) on some metabolic and reproductive profiles in dairy cows during transition period. Pol J Vet Sci 19: 697-706.

Schäfers S, Meyer U, Von Soosten D, Hüther L, Drong C, Eder K, Most E, Tröscher A, Pelletier W, Zeyner A, Dänicke S (**2018**) Influence of conjugated linoleic acids and vitamin E on milk fatty acid composition and concentrations of vitamin A and α-tocopherol in blood and milk of dairy cows. J Anim Physiol Anim Nutr (Berl) 102: e431-e441.

Sharma L, Verma AK, Rahal A, Kumar A, Nigam R (**2016**) Relationship between serum biomarkers and oxidative stress in dairy cattle and buffaloes with clinical and sub-clinical mastitis. Biotechnology 15: 96-100.

Trevisi E, Jahan N, Bertoni G, Ferrari A, Minuti A (**2015**) Pro-inflammatory cytokine profile in dairy cows: consequences for new lactation. Ital J Anim Sci 14: 285-292.

Trevisi E, Zecconi A, Bertoni G, Piccinini R (**2010**) Blood and milk immune and inflammatory profiles in periparturient dairy cows showing a different liver activity index. J Dairy Res 77: 310-317.

Yurdakul I, Aydogdu U (**2019**) The effect of enteritis, pneumonia and omphalitis on oxidative/antioxidant balance in the calves. Turk J Agric Food Sci Technol 7: 539-542.

Zigo F, Elecko J, Vasil M, Ondrasovicova S, Farkasova Z, Malova J, Takac L, Zigova M, Bujok J, Pecka-Kielb E, Timkovicova-Lackova P (**2019**) The occurrence of mastitis and its effect on the milk malondialdehyde concentrations and blood enzymatic antioxidants in dairy cows. Vet Med 64: 423-432.